The crystallization of cellulose.

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Previous investigation.

In number two of volume nine of *La Cellule*, Eugene Gilson of the University of Gand has an interesting article on "The crystallization of cellulose and the chemical composition of the cell membrane of plants."

I will give an abstract of the article and then have something to say of some work in the same line done in the winter and spring of 1894 in the biological laboratory of Johns Hopkins University.

The author first sets forth the present state of our knowledge of the constitution of the cell wall, taking the work of Schultze as the most authoritative. Schultze holds that we have in the cell wall dextrocellulose and mannosocellulose. These two agree in being insoluble in dilute acids or alkalies by boiling and in their coloration by iodine and sulfuric acid, but differ in their derived sugars, the first giving only dextrose and the second both dextrose and mannose. Besides these he proposes to give the name of hemicelluloses to those as yet little known compounds occurring in the cell wall that are soluble in dilute acids and alkalies by boiling, and which probably agree with dextrocellulose and mannosocellulose in their reaction with chloriodide of zinc and iodine and sulfuric acid.

None of these bodies, though closely related to the crystalline sugars, have hitherto been considered to be crystalline but rather amorphous.

Gilson hoped to settle the following points: (*a*) Which of the carbohydrates is it that crystallizes from a solution of the material of vegetable tissue in Schweizer's reagent, when strong ammonia is added? (*b*) Is cellulose a distinct chemical individual or are there several compounds in the cell wall that are insoluble in dilute acids and alkalies, and give the blue color with the iodine reagents? (*c*) Is the cellulose free or in combination in the cell membrane with other constituents? (*d*) Is the cellulose distributed through all three layers of the cell membrane or localized in one or two or in certain parts of all?

In his discussion the author uses the term cellulose to designate provisionally the carbohydrate or carbohydrates of the cell wall that are insoluble in acids and alkalies and give the blue color with iodine and sulfuric acid.

For the author's method of obtaining the so-called crystals of cellulose, we may describe the course pursued for the root of the beet (*Beta vulgaris*), which is only modified in individual cases depending upon the resistance of the tissue at hand. Moderately thick sections of the swollen root were — (1) soaked in 1% KOH (or eau de javelle) to dissolve out the cell contents. In repeating his experiments in the Johns Hopkins laboratory half an hour in 1.5% KOH was generally found sufficient. (2) After thoroughly washing in distilled water the sections were put into Schweizer's reagent for four or five hours. (3) The dissolved cellulose is precipitated in crystals by putting the sections after removal from Schweizer's reagent into ammonia, the size of the crystals being said to vary with the strength of the ammonia, 5% giving only spherocrystals, and 20% giving beautiful arborescent or radially arranged spicular crystals.

These bodies, which have the appearance of crystals under the microscope, can be seen best after clearing the sections of copper compounds by washing them with water and treating with dilute HCl and then coloring them with chloriodide of zinc (or Congo red before treatment with HCl). They are then found almost entirely within the cell walls, sometimes in the intercellular spaces, those that are formed outside being from the cells opened in cutting the section, very little of the dissolved cellulose diffusing through the cell wall. If the Schweizer's fluid has acted long enough the iodine reagents give no evidence of any cellulose remaining in the cell membrane, the only blue coloring substance being the crystals within the cell cavity.

It is from the beet evidently that Gilson gets his most characteristic crystals, as it is these that he figures for his article, though he has worked in this way upon sections of more than fifty plants. Most of these are angiosperms, but among them are chara and spirogyra of the algæ, and mucor and agaricus of the fungi, with half a dozen mosses and ferns and two gymnosperms. In all of these, except the fungi, he was able to get satisfactory crystals of cellulose, and has also obtained good results with a number of seeds, such as coffea,

strychnos, etc., by first treating oily seeds with ether. Cotton fibers and lignified tissues also yield crystals. Another interesting result is the obtaining of crystals from the test of an ascidian, Phallusia mamillata, this being confirmatory of the work of Winterstein who found that tunicine or animal cellulose gave dextrose as a derivative sugar just as vegetable cellulose does, and thus seeming to show that animal and vegetable cellulose are one and the same chemically.

From the results of his work on the plants, the following conclusions are drawn:

1. All that portion of the cell wall that is colored blue by chlorozinc-iodide, and that only, goes to make up the crystals.

2. The crystals are of pure cellulose.

3. All the cell walls contain cellulose, but while the internal layer consists almost entirely of it, the intermediate has only a small proportion, and the intercellular layer only a trace. These three layers were distinguished in sections treated by the method of Mangin, and those stained with methylene blue.

4. That the cellulose is found crystallized within the cell shows that its solution in Schweizer's fluid is not very diffusible, and also gives additional proof that it is derived from the inner layer of the cell-wall.

In the strictly chemical portion of his work, Gilson prepared convenient quantities of cellulose for working with by scraping to a pulp the stalk of cabbage or root of the beet, and proved by putting sections through the same treatment that he had a substance identical with the crystals found within the cells as described above. In fact he got from the pulp a mass of spherocrystals of the same kind formed in the cells.

By the method of Flechsig he finds that when all operations are carried on with extreme care, dextrose only (in the form of the silver salt of the derived saccharic acid) is obtained from cellulose, showing thus that cellulose is a definite chemical individual.

By using the method of Schultze he got from *Coffea arabica* a body corresponding to the mannosocellulose of Schultze. By precipitation from a solution of this with CO_2 he gets characteristic cellulose, and finds another carbohydrate is still left in solution. This second body gives not dextrose but mannose as a derived sugar. Mannosocellulose is then only a

mixture of true cellulose and another carbohydrate which Gilson proposes to call paramannane. The formula of the latter as he determined by several analyses is $(C_{12} H_{22} O_{11})_n$.

The paramannane thus obtained was in the form of a white powder of small spherocrystals, which dissolves in Schweizer's fluid as we have seen, and also in H_2SO_4, in the cold when concentrated, but when dilute only by heating, and then it turns to mannose. Gilson does not state whether paramannane turns blue on the addition of iodine reagents.

The results of his chemical work are: (1) Cellulose is a single chemical individual. (2) Mannosocellulose is a mixture of cellulose and paramannane. (3) The so-called reserve-cellulose is probably also a mixture of cellulose and some other carbohydrate or carbohydrates.

Additional investigation.

At the suggestion of Dr. J. P. Lotsy I repeated much of Gilson's work as given in the first part of his paper, in the laboratory of the Johns Hopkins University, at Baltimore.

I obtained a considerable number of the green plants mentioned by Gilson. From these in every case where good material was at hand the so-called crystals were readily obtained. Beta vulgaris gave very fine crystals after three hours or less in Schweizer's fluid, but a longer time was needed to dissolve all of the cellulose out of the cell wall.

Among others dahlia, lactuca, typha, ceratozamia, equisetum, and chara gave especially good results.

As to the diffusibility of the dissolved cellulose, I found that after keeping sections of the beet in Schweizer's fluid for twenty days, the crystals were as plentiful in the cells as at first.

The solution then resembles in diffusibility rather non-crystalline colloidal substances than crystalline ones.

This together with the appearance of the bodies themselves in some cases, led me to think that the things which Gilson called crystals were not really such. I therefore made several sets of preparations in which there were plenty of the bodies present, and examined them carefully under the polariscope. *In no case did they have any effect on polarized light, showing thus that they were not true crystals.*

I was gratified, though greatly surprised, to find later, that Gilson, after calling the bodies crystals all through his paper

and saying nothing of having tested them with polarized light, gave expression to the conclusion at which I had arrived, in the following sentence *in fine print at the end of the explanation of the plates:* "Les formes sous lesquelles se présentent le paramannane et la cellulose sont sans action sur la lumière polarisée; ce sont donc plutôt des cristallites que des cristaux."

As mentioned in the abstract of Gilson's paper, he got no *crystallites* (as we shall now call them) from mucor and agaricus. I tried both of these, together with saprolegnia and the fungal portions of several lichens, but although they gave the blue color with the iodine reagents, after treating with potash, they did not give any crystallites. The Schweizer's reagent did not dissolve out the blue coloring constituent of the tissues at all, the addition of chloriodide of zinc giving as strong a color after soaking for two weeks in Schweizer's reagent as at first, the color always being evenly diffused through the tissue.

In regard to vegetable tissues then, my work has been entirely confirmatory of Gilson's.

As mentioned above, the only animal tissue with which Gilson worked was the test of phallusia, and here he states that he obtained crystallites similar to those in vegetable cells.

I also worked on several ascidians, beginning with salpa, but although the test gave a strong blue color with chlorozinc iodide both before and after treatment with Schweizer's fluid, I was never able to get any of the crystallites to form, and also, as in the fungi mentioned above, none of the blue coloring substance dissolved out by prolonged treatment with Schweizer's reagent. There were in some cases bodies which I at first mistook for the crystallites, but on more careful examination it was found that their seeming blue color was due to the color of the surrounding tissue, and moreover they proved to be true crystals when tested with the polariscope.

I decided to try Gilson's method in testing for cellulose on other animal tissues and found many suggestions as to what to use in a paper by H. Ambronn, on "Cellulose Reaction bei Arthropoden and Mollusken," in Mittheil. aus der Zool. Sta. zu Neapel **9:** 475.

Ambronn found that after treatment with alcoholic potash he could get a distinct blue with iodine reagents in several widely separated arthropods, such as eupagurus, squilla, sap-

pharina, calotermes and julus. Among the molluscs he obtained the cellulose reaction in sepia, the pen of loligo, the radula of helix and the opercula of several species of natica.

The skeletons of rhizopods among the protozoans, the perisarc of hydroids among the coelenterates, the tubes of annelids such as onuphis and polyodontes and also the skeletons of bryozoans among Vermes, gave no indication of the presence of cellulose.

Without attempting to verify all of Ambronn's results, I worked on a number of animal tissues, first trying the iodine test for cellulose on various parts of different arthropods and mollusks and then with such tissues as the skeleton of horny sponge, integument of the starfish and nereis, and vertebrate dermal tissues such as hair, skin, and nails. In the mollusks the pen of loligo and radula of paludina, and among the arthropods the integument of the abdomen of eupagurus, the stalk of lepas, gills of limulus, and tests of ascidians such as salpa, molgula and amaroecium, all gave results confirmatory of those of Ambronn. As was to be expected from the lack of mention of such a thing in the literature, the vertebrate tissues gave no indication at all of cellulose.

I next applied Gilson's test for the presence of cellulose to some of the above which gave the strongest color with chlorozinc iodide, to see if I could obtain the blue-staining substance in the form of crystallites. The pen of loligo gave an intense violet with chlorozinc iodide but keeping for fifteen or twenty days in Schweizer's fluid did not decrease the intensity of coloration when the sections were stained with chlorozinc iodide afterward, nor could any crystallites be obtained by precipitating with ammonia.

In the same way the tissues of eupagurus, molgula and paludina were tried and all gave results like that in the case of loligo, the blue-coloring substance not seeming to be dissolved out but remaining as at first uniformly diffused through the tissues.

The occurrence of the blue coloration in so many of the truly chitinous animal tissues suggested the idea that chitin or some derivative of it might give the blue color. The method of preparation of glycosamine by Ledderhose resembles in several steps that of Ambronn for the demonstration of cellulose in animal tissues. I therefore (although a blue staining with the iodine reagents had not been mentioned as one

of its properties) made a preparation of glycosamine by the method of Ledderhose, and tried it with chlorozinc iodide to see whether it gave the blue color, and found that it did not.

This subtance is dextro-rotatory and reduces Fehling's solution as readily as dextrose, and these facts may account for the supposed derivation of true dextrose from chitinous tissues by the older observers, and hence we have only the work of Winterstein on tunicine to prove the existence of true cellulose in animal tissues.

In conclusion then, I am inclined to think that Gilson's test for cellulose is a much more satisfactory one than the ordinary chlorozinc iodide test, and should therefore replace the old method in the demonstration of cellulose in vegetable tissues in the laboratory. Its use in my hands seems to prove that the blue staining substance present in tunicates, arthropods and certain mollusks is not identical with vegetable cellulose chemically.

We must, however, consider the animal substance under discussion as possibly quite similar to, perhaps only a slight modification of, true cellulose. For as we have seen above, the substance of the supporting tissue of the fungi, which as we know have arisen from plants that in all probability possessed true cellulose, are still as refractory as animal tissues when treated with Schweizer's solvent.

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